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Long-term stability of the inversion process for sugar and ethanol production in an existing Japanese sugar mill

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Abstract In order to utilize sugarcane with a high reducing-sugar content as the raw material for sugar production, a new technology called the 'inversion process' has been developed. This new technology aims to enhance raw sugar yield via removal of reducing sugars through selective ethanol fermentation using an invertase-defective yeast, prior to sugar crystallization. To assess the feasibility of the inversion process technology in an existing sugar mill, a test of continuous and repeated-batch fermentation using clear juice was undertaken at a pilot-scale facility at the Shinko Sugar Mill in Japan. Batch fermentation trials were performed at 35°C for 1.5-3 h and repeated 70 times in a 2000 L fermenter using the same culture of invertase-defective yeast strain GYK-10. To confirm the long-term stability of the selective fermentation, the concentrations of saccharides and ethanol in the fermenter were measured every hour and the residual sucrose ratio and the reducing sugars removal ratio were calculated. Each batch was checked for the presence of contaminating bacteria. The results showed that 88.4% of reducing sugars were converted to ethanol and 99.5% of sucrose remained throughout the 70 consecutive batch fermentations. This indicates that the saccharometabolism selectivity of GYK-10 is stable. Although contamination by some microorganisms, such as *Clostridium beijerinckii*, *Bacillus simplex*, and *Bacillus brevis*, was observed, this had little influence on the fermentation outcomes. This paper reports on the feasibility of using the inversion process in an existing sugar mill.

Key words Inversion process, selective fermentation, reducing sugar, invertase-defective yeast, long-term stability

INTRODUCTION

Reducing sugars (RS; i.e., glucose and fructose) in sugarcane juice increase syrup viscosity and inhibit crystallization of sucrose (Hook 1946). Recently, a new technology to selectively remove RS from sugarcane juice, the 'inversion process', has been developed (Ohara *et al*. 2012).

In the inversion process, RS in sugarcane juice is selectively converted to ethanol using an invertase-defective yeast prior to sucrose crystallization. In general, most yeast strains produce invertase, an enzyme that converts the disaccharide sucrose into monosaccharide RS outside the cell resulting in the conversion of sucrose to ethanol. In contrast, invertase-defective yeast cannot produce or release invertase from the cell and, therefore, ferments only the RS and leaves sucrose untouched. This new process inverts the traditional production sequence of sugar and ethanol (Fig. 1), hence the term 'inversion process'.

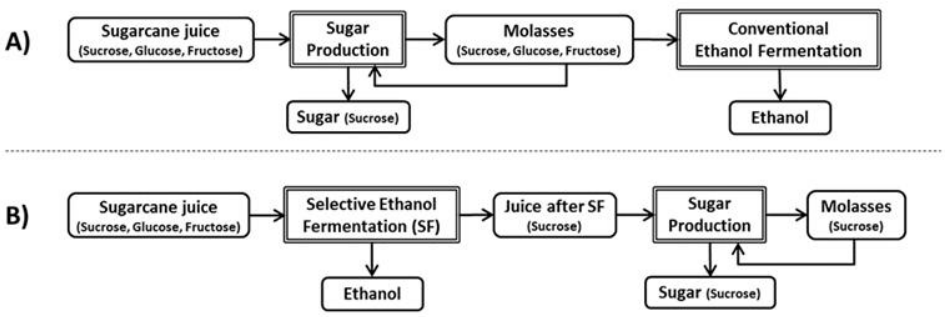

Fig. 1. Schematic of sugar and ethanol production from sugarcane juice: A) conventional process; B) inversion process.

Regardless of the level of RS in sugarcane, the inversion process removes the RS by converting it into ethanol, thereby allowing more efficient production of raw sugar. This process facilitates the utilization of sugarcane with high RS contents, such as premature and over-mature crops, or high-productivity cultivars, and may also allow more intensified use of land and the extension of the operating period of sugarcane mills. Furthermore, the inversion process can be introduced into traditional sugar processing simply by adding a fermentation tank containing the invertase-defective yeast, thereby increasing syrup purity and resulting in the simultaneous production of ethanol and sugar.

A laboratory-scale example of the inversion process using the invertase-defective yeasts *Saccharomyces dairenensis*, *S. transvaalensis*, *S. rosinii* and *Zygosaccharomyces bisporus* has been reported (Ohara *et al*. 2013). However, the commercial implementation of this new process in existing cane sugar mills requires a yeast strain that: (1) can be used in food production processes; (2) exhibits stable saccharometabolic selectivity; and (3) can be easily separated from the saccharide solution. Recently, *S. cerevisiae* GYK-10 strain, which is available for food production, has been developed as a suitable strain for the inversion process (Kato *et al.* 2016). GYK-10 has the characteristics of being both sucrose utilization negative and flocculating, because it was developed by interbreeding a strain that lacked the capacity for sucrose utilization with a flocculent strain.

However, there are three other practical obstacles to the commercial implementation of this process. The first is the potential lack of stability of saccharometabolic selectivity during multiple consecutive utilizations of the yeast. The second is that GYK-10 may lose its flocculating characteristics during long-term use. If GYK-10 loses its flocculating characteristics, an additional yeast separator must be employed after the fermentation reactor, which increases the cost. The third obstacle is the potential for contamination of the syrup by harmful microorganisms. Although clear juice (CJ) is heated to more than 100°C, some types of spore-forming bacteria are reported to survive in high-temperature environments in the form of spores. Therefore, there is potential for an increase in bacteria in the fermenter under long-term operation.

To assess the feasibility and long-term stability of the inversion process technology, we conducted a continuous and repeated-batch fermentation test of GYK-10 using CJ from Shinko Sugar Mill (Kagoshima, Japan).

MATERIALS AND METHODS

Sugarcane juice

Juice was obtained continuously via a bypass line from a CJ pipe in the Shinko Sugar Mill. The sugarcane juice was mainly derived from NiF8 (*Saccharum* spp. hybrid), which is the leading variety used for raw sugar manufacture in Japan. Brix, sucrose, and RS contents in the CJ were 9.7-13.1°Brix, 0.79-1.10 g/L, and 0.04-0.07 g/L, respectively.

Microorganism and culture conditions

The invertase-defective yeast used in this study was the strain *S. cerevisiae* GYK-10, which was developed as described above. Yeast culture was performed as described by Kato *et al*. (2016).

Selective fermentation test at pilot-scale plant in an existing sugar mill

The repeated-batch fermentation test using CJ from Shinko Sugar Mill was conducted at a pilot-scale test facility (Fig. 2). Batch-fermentation experiments were performed at 35°C for 1.5-3 h and repeated 70 times. CJ (95°C) was cooled to 35°C by a heat exchanger and sent to each fermenter. An antimicrobial agent, BetaStab 10A (Beta Tec, Germany), was added (0.15 g/L on average) to the CJ at every batch from the batch 1 to 50. Then 150 L of yeast broth was inoculated into 750 L of juice in each 2000 L fermenter so that the initial cell concentration reached 1.5×10^7 cells/mL The total working volume of the fermenter was 900 L. After fermentation, the fermented juice was allowed to rest for 10-30 min for the flocculent yeast to settle by gravity. Next, 750 L of supernatant was pumped from the fermenter via a side pipe and 150 L of settled yeast remained in the bottom of the fermenter which was used for the subsequent fermentation without washing. This procedure was repeated 70 times in each fermenter. Batch schedules (Fig. 3) were changed according to the five stages as shown in Table 1.

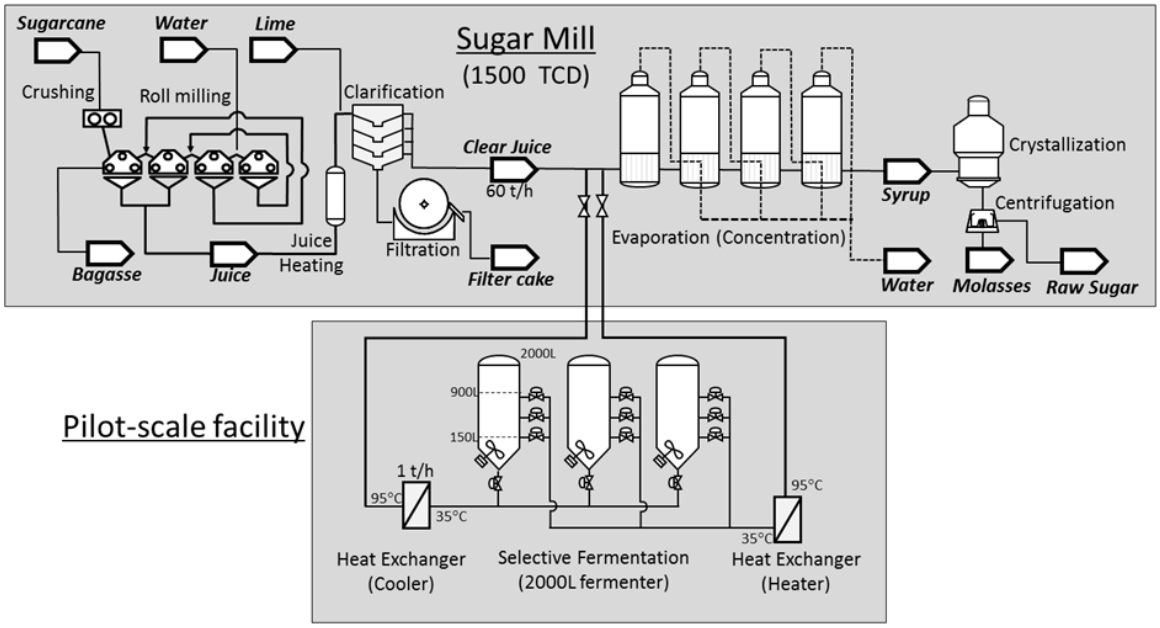

Fig. 2. Process flow of the pilot-scale fermentation facility.

Table 1. Batch schedules for the five stages.

Process		Batch No. 1-20	Batch No. 21-52	Batch No. 53-70
(1) Receiving juice	[min]	60	40	30
(2) Fermentation	[min]	60	40	20
(3) Still standing	[min]	30	10	10
(4) Withdrawal	[min]	20	20	20
(5) Standby	[min]	10	10	10
Total time	[min]	180	120	90

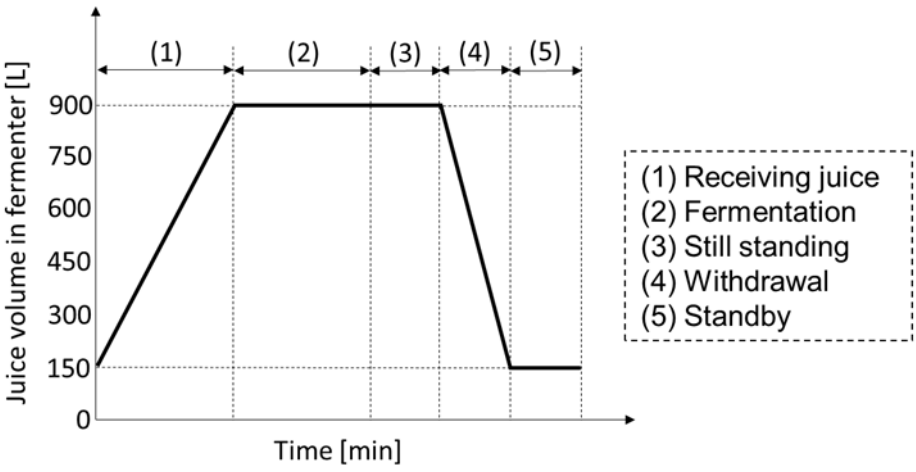

Fig. 3. Diagram of batch schedule.

Confirmation of long-term stability

Stability of the saccharometabolic selectivity of the yeast

To confirm the long-term stability of the saccharometabolic selectivity of GYK-10 strain, the concentrations of saccharides (i.e., sucrose, glucose, and fructose) and ethanol in the fermenter were measured every hour and then we calculated the residual sucrose ratio, the RS removal ratio, and ethanol yield. The residual sucrose ratio was calculated as the amount of sucrose in the fermented juice after each batch as a percentage of the sucrose in the CJ before each batch. RS removal ratio was calculated as the amount of RS consumed during each batch as a percentage of the amount of RS before each batch. The ethanol yield was calculated as the amount of ethanol produced derived from the measured ethanol concentration, as a percentage of the theoretical amount, where we assumed that the RS in the CJ were completely converted into ethanol.

Stability of the flocculating characteristics of the yeast

To check the long-term stability of the flocculating characteristics of the yeast, the yeast concentration of the supernatant after each fermentation was measured. The yeast concentration was calculated as the amount of yeast after centrifugation ($5000 \times g$, 5 min) as a percentage of the amount in each supernatant sample.

Microbiological stability in the fermenter

To confirm the microbiological stability in this system, the presence or absence of bacteria in the CJ and supernatant after fermentation was checked. Microbiological identification of the CJ was performed every 5 h and of the supernatant after every five consecutive batch fermentations. Liquid samples for bacteriological tests were diluted according to the serial dilution method. For detection of anaerobic and aerobic bacteria, the diluted samples were spread on de Man, Rogosa, and Sharpe agar medium (MRS medium: De Man *et al*. 1960) and standard agar medium, respectively. The anaerobic and aerobic plates were incubated at 37°C for 48 h under anaerobic and aerobic conditions, respectively. Identification of the isolated bacteria was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS: Schubert *et al*. 2011) and 16s rRNA gene sequence methods (Lane *et al*. 1985). MALDI-TOF MS was measured via Microflex LT mass spectrometer and MALDI Biotyper software (Bruker Daltonics, Germany).

Analytical methods

Sugar composition was measured via high-performance liquid chromatography using an LC-10AD chromatogram fitted with an RID-10A refractive index detector (Shimadzu, Japan). Sugars were separated on a SUGAR SC1011 column (Shodex, Japan) using distilled water as the mobile phase at a flow rate of 0.8 mL/min. Column temperature was maintained at 80°C. Ethanol concentration was measured using a BF-5 biosensor (OSI, Japan).

RESULTS AND DISCUSSION

Long-term stability of the saccharometabolic selectivity of the yeast

Figure 4 shows the indicators of selective fermentation performance, i.e., the residual sucrose ratio, the RS removal ratio, and ethanol yield, obtained in 70 consecutive repeated pilot-scale batch fermentations.

The residual sucrose ratio remained around 100% throughout the tests and averaged 99.5% for the 70 batches. We saw no significant degradation or consumption of sucrose caused by selective fermentation for any of the batches. The residual sucrose ratio was sometimes greater than 100% because the sucrose concentration of the CJ measured at one time point before each batch fermentation was used as a substitute for the sucrose concentration of the total CJ at the beginning of fermentation. The RS removal ratio and the ethanol yield remained at high levels even during the last half of the batch fermentations and averaged 88.4% and 81.1%, respectively, for the 70 batch fermentations. Therefore, the GYK-10 strain demonstrated long-term stability of its saccharometabolic selectivity in the existing sugar mill for at least 70 batches.

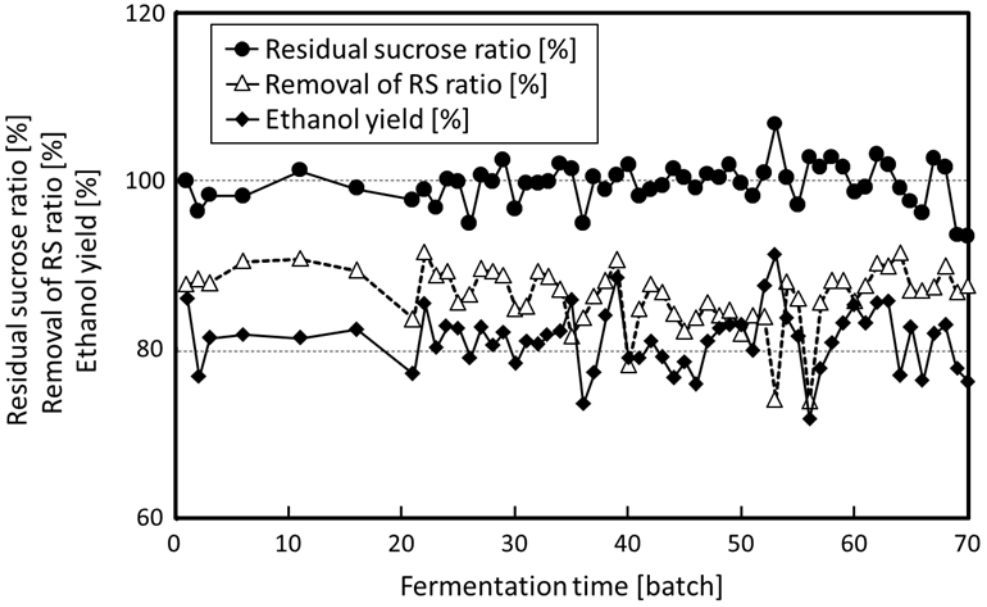

Fig. 4. Profile of fermentation indicators in 70 continuous batch fermentations.

Some invertase-defective yeasts have been previously reported to restart utilizing sucrose for several reasons, e.g., by taking sucrose into the cell in conditions where RS was depleted (Badotti *et al*. 2008). We supplied fresh CJ immediately after fermentation, i.e., at the depletion of RS, because the total batch fermentation lasted for only a short time (1.5-3 h). Therefore, no degradation of sucrose should have occurred.

Long-term stability of the flocculating characteristics of the yeast

Figure 5 shows the profile of yeast concentrations in 70 consecutive repetitive batch fermentations. The yeast concentrations in the supernatants were consistently less than 0.2% (w/w) except for batches 53-55. In these batches, yeast flowed out of the fermenter at around 0.4% (w/w), but this is still quite a low level compared with the yeast concentration in the fermenter. Therefore, there was no effect on the flocculating capacity of GYK-10 strain during the 70 consecutive batch fermentations using an actual CJ.

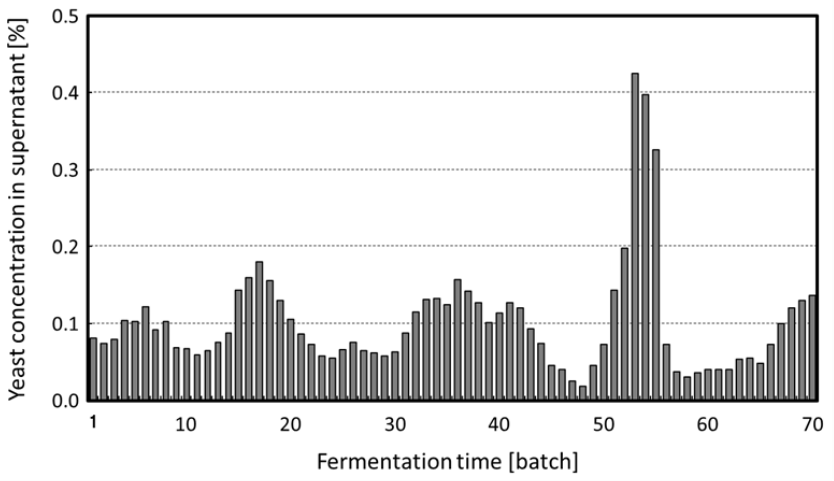

Fig. 5. Profile of yeast concentrations in the supernatant during long-term fermentation.

It is quite unlikely that the cause of the yeast outflow was the loss of the flocculent ability of the yeast because the supernatant contained a certain level of yeast even at an early stage. From our observations, we inferred that the yeast outflow occurred because of the adhesion between the yeast and a floating scum. The scum was thought to accumulate over the repetitive batch fermentation. Therefore, it may be possible to reduce the outflow of yeast by spraying water from the top of the fermenter to prevent the generation of scum.

Microbiological stability in the fermenter

The results of microbiological analysis of CJ and the supernatants after fermentation (Fig. 6) indicated that: (1) some bacteria were detected in the CJ although at quite a low level; (2) there was no contamination in the supernatant at the early stages; and (3) some bacteria were detected at around 10^3 cells/mL from the middle stages of batch fermentation.

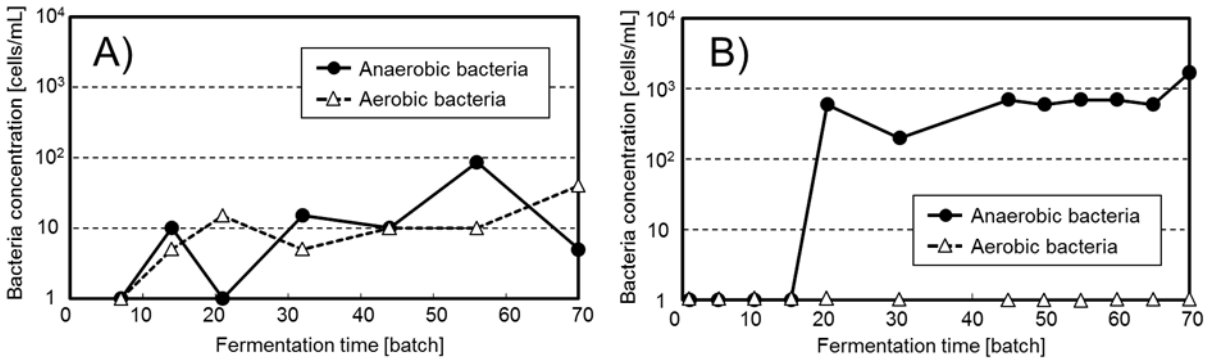

Fig. 6. Microbiological analysis of: A) CJ and B) supernatant after fermentation.

The bacteria isolated from CJ and the supernatant were identified by MALDI-TOF MS and 16s rRNA gene sequence methods (data not shown). The bacteria isolated from CJ were *Clostridium beijerinckii* in most batches and *Bacillus simplex* only in batch 70. Only *C. beijerinckii* was isolated from any batch supernatant. These bacteria are reported to be spore-forming types of bacteria that tolerate high temperatures. However, Figures 4 and 5 show that *C. beijerinckii* is not a harmful bacterium because there was little change in the fermentation despite the contamination.

Lactobacilli were not detected in this experiment. *Lactobacillus* is known to be a harmful bacterium for fermentation because it has a high growth potential and likes a similar growth environment to yeast. Therefore, some countermeasures against contamination, for example, regular cleaning of tanks and lines, are necessary. However, it is time-consuming and costly to detect bacteria. The best means to quickly and simply monitor and control contamination remains an issue.

CONCLUSION

We have shown that the GYK-10 strain exhibited complete saccharometabolic selectivity and sustained flocculation throughout 70 consecutive fermentation tests using actual CJ from an existing sugar mill in a pilot-scale fermenter.

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Stabilité à long terme de la procédure d'inversion pour la production de sucre et d'éthanol dans une sucrerie japonaise

Résumé. Afin d'utiliser la canne à sucre avec une forte teneur en sucre réducteurs comme matière première pour la production de sucre, une nouvelle technologie appelée le « processus d'inversion » a été mise au point. Cette nouvelle technologie a pour but d'améliorer le rendement du sucre brut par la suppression des sucres réducteurs et par une fermentation sélective d'éthanol à l'aide d'une levure ne contenant pas d'invertase, avant la cristallisation du sucre. Afin d'évaluer la faisabilité de cette technologie, des essais de fermentation continues et discontinues à partir du jus clair ont étés entrepris dans une installation pilote à l'usine de Shinko au Japon. Des essais de fermentations discontinues ont eu lieu à 35°C pendant 1,5 à 3 h et répétés 70 fois dans une cuve de 2000 L à l'aide de la même culture de levure GYK-10 sans invertase. Pour confirmer la stabilité à long terme de la fermentation sélective, les concentrations de saccharides et d'éthanol dans la cuve de fermentation ont étés mesurées toutes les heures et on a calculé le ratio de saccharose résiduel et le ratio de suppression des sucres réducteurs. La présence de bactéries a été déterminée dans chaque essai. Les résultats ont montré que 88,4 % des sucres ont été convertis en éthanol; 99,5% du saccharose est retrouvé après les70 fermentations en discontinue. Cela indique que la sélectivité de la GYK-10 est stable. Bien que la contamination par des micro-organismes, comme le *Clostridium beijerinckii, Bacillus simplex* et le *Bacillus brevis*, a été observée, cela a eu peu d'influence sur les résultats de la fermentation. Cet article décrit la possibilité d'utiliser le processus d'inversion dans une sucrerie.

Mots-clés: Processus d'inversion, fermentation sélective, sucres réducteurs, levure sans invertase,, stabilité à long terme

Estabilidad a largo plazo del proceso de inversión para la producción de azúcar y etanol en un ingenio existente en Japón

Resumen. Con el fin de emplear caña de azúcar con un elevado contenido de azúcares invertidos como materia prima, para la producción de azúcar, se ha desarrollado una nueva tecnología denominada "proceso de inversión". Esta nueva tecnología esta dirigida al propósito de estimular el rendimiento de azúcar crudo, mediante le remoción de de los azúcares reductores a través de una fermentación alcohólica selectiva empleando una levadura invertasa- deficiente, previa ala cristalización del azúcar. Para alcanzar la factibilidad de la tecnología del proceso de inversión en un ingenio azucarero existente, se realizó un trabajo experimental de fermentación continua y en batch repetitiva, empleando jugo clarificado, en una instalación piloto de Shinko Sugar Mill en Japón. Las prueba de fermentación batch se realizaron a 35°C por 1.5-3.0 horas y repetidas 70 veces en un fermentador de 2000 L, empleando el cultivo de la misma cepa de levadura invertasa-deficiente GYK-10. Para confirmar la estabilidad a largo plazo de la fermentación selectiva, se midió la concentración de sacáridos y etanol en el fermentador cada hora, y se calculó la relación de sacarosa y la razón de remoción de azúcares. En cada templa se chequeó la presencia de bacterias contaminantes. Los resultados muestran que el 88.4% de los azúcares reductores se convirtieron en etanol y el 99.5% de la sacarosa se mantuvo a lo largo de las 70 templas consecutivas. Esto indica que el metabolismo sacarífero selectivo de la cepa GYK-10 es estable. Además, se observó la contaminación con algunos microorganismos como el *Clostridium beijerinckii, el Bacillus simplex y el Bacillus brevis* tenían poca influencia, en los resultados de las fermentaciones. Este trabajo reporta la factibilidad del empleo del proceso de inversión en un ingenio azucarero existente.

Palabras clave: Proceso de inversión, fermentación selectiva, azúcares reductores, levadura invertasa-deficiente, estabilidad a largo plazo